PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
SP/SC/N8862		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 03316	08/10/1999	09/10/1998
Applicant		
KING'S COLLEGE LONDON et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/03316

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claim 14 (partially, as far as an in vivo method is concerned) is directed to a diagnostic method practised in the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 15-16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claim 14 (partially, as far as an in vivo method is concerned) is directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
Although claims 15-16 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03316

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C12N15/12 G01N33/68 C12N15/70 A61K38/17
C12N1/21 A61P37/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 08012 A (REESEARCH CORPORATION TECHNOLOGIES) 14 April 1994 (1994-04-14) claims 3,7,8 page 1, line 13 - line 15	3,4,8-13
X	TING J ET AL: "Human gene encoding the 78,000-dalton glucose regulated protein and its pseudogene: structure, conservation and regulation." DNA, vol. 7, no. 4, 1988, pages 275-86, XP000877141 abstract	3,4,8-13

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 2000	15/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Flao, K

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEBORGNE-CASTEL N ET AL: "Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress." THE PLANT CELL, vol. 11, no. 3, March 1999 (1999-03), pages 459-70, XP002131460 abstract	17
P,X	ST. BLASS (1) ET AL: "The rheumatoid arthritis -associated autoantigen p68 identified as heavy chain binding protein (BIP." ARTHRITIS & RHEUMATISM, (SEPT., 1999) VOL. 42, NO. 9 SUPPL., PP. S244. MEETING INFO.: 63RD ANNUAL SCIENTIFIC MEETING OF THE AMERICAN COLLEGE OF RHEUMATOLOGY AND THE 34TH ANNUAL SCIENTIFIC MEETING OF THE ASSOCIATION OF RHEUMATOLOGY HEALTH PROFESSIONAL, XP002131445 abstract	1-12, 14-16
P,X	WO 99 18131 A (BLÄSS S) 15 April 1999 (1999-04-15) cited in the application claims 1-15	1-9,14
P,X	WO 99 53040 A (METAGEN GESELLSCHAFT FÜR GENOMFORSCHUNG) 21 October 1999 (1999-10-21) page 108	10-13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/03316

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9408012 A	14-04-1994	EP 0663010 A	19-07-1995
		US 5773245 A	30-06-1998
WO 9918131 A	15-04-1999	DE 19744132 A	22-04-1999
WO 9953040 A	21-10-1999	DE 19817557 A	21-10-1999

PATENT COOPERATION TREATY

PCT

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
IT/GM/N8862		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/03316	08/10/1999	09/10/1998
International Patent Classification (IPC) or national classification and IPC C07K14/47		
Applicant KING'S COLLEGE LONDON et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05/05/2000	12.01.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Petri, B Telephone No. +49 89 2399 7356

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/03316

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-41 as originally filed

Claims, No.:

1-17 as received on 05/10/2000 with letter of 05/10/2000

Drawings, sheets:

1/3-3/3 as originally filed

Sequence listing part of the description, pages:

1-8, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 15, 16.

because:

   ☒ the said international application, or the said claims Nos. 15, 16 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-3, 5-16
	No:	Claims	4, 17
Inventive step (IS)	Yes:	Claims	1-2, 4-7, 9, 11, 14-16
	No:	Claims	3, 8, 10, 12-13
Industrial applicability (IA)	Yes:	Claims	all
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claims 15-16 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
Reference is made to the following document/s/:

D1: TING J ET AL: 'Human gene encoding the 78,000-dalton glucose regulated protein and its pseudogene: structure, conservation and regulation.' DNA, vol. 7, no. 4, 1988, pages 275-86, XP000877141

D2: WO 94 08012 A (REESEARCH CORPORATION TECHNOLOGIES) 14 April 1994 (1994-04-14)

Novelty; Art 33(2), PCT

2. Although immunoglobulin heavy chain binding protein BiP(78kD) (=GRP70) is known (see e.g. GENBANK Accession No X87949; D1: page 2, line 10) the use of BiP as a reagent for indicating the presence of RA, tests comprising BiP, Bip and its DNA for use in therapy, methods for testing for RA, and methods of therapeutic treatment is not disclosed in the available prior art. Consequently subject-matter of invention claims 1-2, 5-11, 14-16 is considered novel.

   In addition, as Seq. Id. Nos 1-3 are different from the sequences of BiP in the available prior art, subject-matter of claims 2-3, 12-13 is also considered novel.

3. However subject-matter of claims 4 and 17 relating to recombinant BiP and

organisms is considered to lack novelty in view of e.g. D2, wherein BiP is overexpressed in yeast.
In addition it has to be noted that the feature "Recombinant" (claim 4) does not impose any limitation on the product of said claim, as BiP obtained by recombinant methods is indistinguishable from BiP obtained by other means. Consequently subject-matter of claim 4 also lacks novelty in view of the known BiP (see item 2).

Inventive Step; Art 33(3), PCT

4. Subject-matter of claims 1-2, 5-6, 9, 11, 14-16 relate to BiP for use in therapy and diagnosis of rheumatoid arthritis. BiP and its function as molecular chaperone in the lumen of the ER was known (see e.g. D1, D2). In addition to that known function the applicants have shown that a particular BiP isolated from chondrocytes possesses characteristics of a rheumatoid autoantigen. This association of BiP with rheumatoid arthritis and consequently the specific therapeutical implication of BiP is not derivable in an obvious way from the available prior art. Consequently, subject-matter of claims 1-2, 5-6, 9, 11, 14-16 is considered to constitute an inventive contribution to the art.

5. Subject-matter of claims 3, 12-13 relates to a novel sequence encoding for a BiP. Although said sequence has been discovered in the course of the unexpected identification of BiP as a rheumatoid autoantigen, the protein itself, i.e. BiP having the sequence as in Seq. Id No 1-3, is considered not to be an inventive contribution to the art.

   The known sequence of BiP is considered the closest prior art. Subject-matter of claims 3, 12-13 differs from said closest prior art in that a BiP with a different sequence is disclosed. As however, no further technical effect is apparent, the technical problem to be solved is considered to be the provision of a further BiP.

   However, to establish inventive activity, the provision of a sequence must be justified by the technical purpose, i.e. by a hitherto unknown or unexpected technical effect, caused by those technical features which distinguish the claimed molecules from other ones. In said sense, the fact that it might have been unexpected to find the particular sequence specifically in a particular cell type or that it was unexpected that a further sequence is naturally occurring in humans is irrelevant for the molecule

itself, as long as these unexpected sources are not reflected in particular technical properties.

Consequently, the provision of the particular sequence with BiP function is only one of many equally suitable solutions, to the above technical problem. Other equally suitable solutions are e.g. further proteins with BiP function which may generated artificially, by modifications that do not affect BiP function. Consequently, the claimed molecules (claims 3, 12-13) are considered to lack an inventive step, pursuant to Article 33(3) PCT.

The applicant may want to argue that the technical problem is to be considered to provide substances for use in treatment and diagnosis of rheumatoid arthritis, and since said use is already unexpected for the prior art members of the BiP family than it is even more unexpected for new members.
However, this consideration is irrelevant for claims relating to the product itself. There are many reasons and motivations prompting the skilled person to generate and/or provide variants of existing proteins. As however the mere provision of further variants itself does not reflect inventive activity, any such new variant has to be justified by a new technical contribution, based on those features that distinguish the molecule from the prior art. In the present case however, said new property is shared by all members of the BiP family.

6. BiP is known to be an important protein conserved throughout evolution and has known biological/physiological functions, e.g. that of a major molecular chaperone of the ER involved in the processing/folding of many disease related proteins such as APP, viral proteins, immunoglobulins, and thyroglobulin. Starting from said known physiological functions, the mere formulation of the idea to use said protein in some form of therapy (claim 8, 10) cannot be considered an inventive contribution to the art, as this implication is highly expected if not predicted by the known physiological functions.
In view of the extensive prior art about the physiological roles BiP plays in cellular function, the actual contribution of the present application is not that for the first time it has unexpectedly realized that this protein is of some therapeutical use, but the particular use of BiP in therapy of rheumatoid arthritis. This is based on the unexpected finding that BiP is a potential rheumatoid autoantigen (see above), which

is not suggested by the known established functions in the context of protein folding.

Any further unexpected implication in other diseases will be based on a similar unexpected association, requiring similar inventive skill. However, as no further such association is disclosed, or any other data support a role of BiP in diseases other than rheumatoid arthritis, any further therapeutical implication of BiP i.e. use of BiP or its DNA for use in any kind of therapy for any kind of disease is considered not enabled and supported (Art. 5/6, PCT).

Re Item VI
Certain documents cited

7. Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO99/18131	14.04.1999	30.09.1998	01.10.1997
WO99/53040	21.10.1999	07.04.1999	09.04.1998

Re Item VIII
Certain observations on the international application

8. For the assessment of the present claims 15-16 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known com- pound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C07K 14/47, C12N 15/12, G01N 33/68, C12N 15/70, A61K 38/17, C12N 1/21, A61P 37/06

A1

(11) International Publication Number: **WO 00/21995**

(43) International Publication Date: 20 April 2000 (20.04.00)

(21) International Application Number: PCT/GB99/03316

(22) International Filing Date: 8 October 1999 (08.10.99)

(30) Priority Data:
9822115.3 9 October 1998 (09.10.98) GB

(71) Applicant *(for all designated States except US)*: KING'S COLLEGE LONDON [GB/GB]; The Strand, London WC2R 2LS (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PANAYI, Gabriel, Stavros [GB/GB]; 14 Westlinton Close, London NW7 1PY (GB). CORRIGALL, Valerie, Mary [GB/GB]; 25 Copley Way, Tadworth, Surrey KT20 5QS (GB). BODMAN-SMITH, Mark, Duncan [GB/GB]; 285a Long Lane, East Finchley, London N2 8JW (GB). FIFE, Mark, Stewart [GB/GB]; 28 Archbishops Place, Brixton, London SW2 2AJ (GB). LANCHBURY, Jeremy, Shaun [GB/GB]; 10 Claremont Road, Tonbridge Wells, Kent TN1 1SZ (GB).

(74) Agents: CRESPI, Romeo, Stefano et al.; Williams, Powell & Associates, 4 St. Paul's Churchyard, London EC4M 8AY (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TREATMENT OF INFLAMMATORY DISEASE

(57) Abstract

The immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom provides a reagent for indicating the presence of rheumatoid arthritis. The protein used is recombinant BiP(78KD) having a defined sequence. A prognostic or diagnostic test for RA may be an ELISA assay or a Western Blot. The invention also includes a method of testing for RA, using a protein BiP(78KD) or a cDNA sequence coding therefor to determine the presence of antibodies to said protein. The invention also includes a method of therapeutic treatment of RA comprising administering the protein BiP(78KD) or a DNA coding therefor e.g. by the intravenous, nasal, oral, or cutaneous route.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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TREATMENT OF INFLAMMATORY DISEASE

This invention relates to inflammatory disease and more particularly to rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints which leads to joint destruction, disability and early death. Although the cause of RA is presently unknown, it has been suggested that type II collagen, uniquely found in the articular cartilage, is a possible autoantigen for RA. It has recently been proposed that gp39, a 39KD glycoprotein, and peptides derived from it, are such autoantigens. However, the data supporting this hypothesis are limited and the role of gp39 therefore remains uncertain.

The present invention stems from a different approach based on a study of chondrocytes, the specialised cells of articular cartilage. We have isolated a protein from human chondrocytes and human chondrosarcoma cell lines which reacts with antibodies present in RA patients' sera and meets the accepted criteria for a putative autoantigen. This purified protein has been tested for proliferation of T cells and has been shown to selectively proliferate synovial T cells from patients with RA. This protein is the immunoglobulin heavy chain binding protein BiP(78KD).

International patent application WO 99/18131 proposes the detection of antibodies to a BiP deriving from Hela cells as a diagnostic indication of RA. However, this prior disclosure does not describe the extraction of BiP from the Hela cells in a reproducible manner and is therefore insufficient for practical application.

We have now obtained and identified the correct RA autoantigen and this discovery leads to the development of prognostic and diagnostic tests for this disease and specific therapy. We have isolated and sequenced the DNA for this protein. We have also cloned and expressed this DNA. The amino acid and DNA sequences are novel and are shown in Sequence Listings appended to this specification. The aminoacid sequences of the BiP protein are given as sequence listings in two versions SE1 and SE2 either of which may be used as a test reagent in accordance with the present invention. The cDNA for SE1 is given as Sequence listing SE3. This sequence has been deposited with GENBANK under Accession No AF 188611.

A comparison of this sequence with that of GENBANK Accession No X87949 is provided hereinafter.

The first part of the following description concerns the characterisation of such an autoantigen; the second the cloning, sequencing and expression of the protein; and the third part the demonstration of disease (rheumatoid arthritis) and tissue (synovial compartment) T cell specificity to the autoantigen.

Part 1: CHARACTERISATION OF AUTOANTIGEN

Chondrocytes/chondrosarcoma cells.

Chondrocytes were isolated from cartilage obtained during joint replacement. The cartilage was minced finely and digested with 1mg/ml collagenase (Worthington). Following digestion the cells were centrifuged at 300g and resuspended in Dulbeccos minimal essential medium (DMEM)(Life Technologies, Paisley, UK) enriched with 10% foetal calf serum (FCS)(Harlan Sera-Lab, Loughborough, UK). Cell debris was washed off the adherent cells after 24hours and the cells allowed to expand until confluent. Cells were passaged using trypsin (0.25%) and split 1:3.

Chondrosarcoma cells (HTB94) (SW1353) were supplied by the American Type Culture Collection ATCC (Rockville, Maryland, USA) and by Dr J Block, Rush University, Chicago, USA (personal gift). These cells were cultured in DMEM with 10% FCS and split 1:3 after gentle trypsinisation (0.25% trypsin Life Technologies, Paisley, UK) when confluent.

Preparation of cell lysates.

Cells were scraped from the flask surface, homogenised and sonicated in the presence of proteinase inhibitors PMSF (2mM), leupeptin (200µg/ml) and aprotinin (50µg/ml) (Sigma, Poole, UK).

Sodium dodecyl sulphate (SDS) (Sigma, Poole, UK) was added to a final concentration of 1% and the proteins solubilised at room temperature for

1hour. Protein concentration was estimated by bicinchoninic acid assay using bovine serum albumin (BSA) as a standard protein (Sigma) and the cell lysate was used at 10µg/well equivalent.

Polyacrylamide gel electrophoresis (PAGE) and Western Blot.

The Mini Protean system (BioRad Laboratories, Hemel Hempstead, UK) was used to run the gels. 5, 7.5 or 10% SDS polyacrylamide denaturing gels 1.5mm thick were prepared (See Appendix 1). Gels were loaded with 10µg protein/well or the equivalent was loaded on preparative gels. Electrophoresis was carried out at a constant 100V and broad range kaleidoscope markers (BioRad) were run in parallel with the cell lysates.

Following electrophoresis the proteins were blotted onto nitrocellulose at a constant 100V for 1hour (See Appendix 1). Nitrocellulose was then blocked with 3%BSA (Sigma, Poole,UK) and left at 4^0 C overnight. Preparative gel membranes were cut into 16 thin strips when necessary, each having an identical protein profile. The membranes were probed with patients sera (1/100 dilution) or specific monoclonal antibodies (at required concentrations) for 1hour at room temperature and then washed x3 over 1hour in TTBS (See Appendix 1). The secondary antibody, goat anti-human IgG (Fab^2) horse radish peroxidase(HRP) conjugate (Sigma) was added at 1/1000 dilution and incubated for 1hour at room temperature. The membranes were then washed x3 over 1hour in TTBS. Enhanced chemiluminescence (Amersham) was used to develop the system and antigen-antibody.HRP complexes appeared as discrete bands on photographic film when developed.

Isolation of the putative autoantigen p78

The band of interest was seen in approximately 30% of the rheumatoid arthritis sera used to screen the cell lysates as previously described.

To isolate the protein the cell lysate was concentrated x 23, using a 30,000MW cut-off filter(Vivascience). This protein was then loaded on 5% and 7.5% gels in parallel . One lane on each was loaded at normal concentration while the two other lanes were overloaded with the protein. Kaleidoscope markers were loaded on either side of the test lanes. The gels were then run as previously described until the kaleidoscope markers showed that the 70,000 MW protein would be in the bottom third of the gel as close as possible to the run-off point. The gels were then blotted onto PVDF membrane (Immobilon P, Millipore) which was immediately placed in distilled water after transfer of the proteins was complete. The strip with normal loading was used for immunodetection of the protein band. The developed film of this immunodetection and Ponceau red staining of the overloaded strips was used to identify the band on the membrane which was then air-dried.
These strips were then taken to isolate and sequence the protein using matrix assisted laser desorption ionisation (MALDI) spectroscopy .

The electroblotted proteins were stained with Ponceau S (0.05% w/v aqueous methanol/0.1% acetic acid) using a rapid-staining protocol (1). The dried, stained proteins were then digested in situ with trypsin (Boehringer, modified) and the peptides extracted with 1:1 v/v formic acid:ethanol (2). One 0.2 µl aliquot (approximately 5% of the total digest) was sampled and directly analysed by matrix-assisted laser desorption ionisation (MALDI) time-of-flight

mass spectrometry using a LaserMat 2000 mass spectrometer (Thermo Bioanalysis,UK) (3). A second 0.2µl aliquot was quantitatively esterified using 1% v/v thionyl chloride in methanol and also analysed by MALDI to provide acidic residue composition (4). Native and esterified peptide masses were then screened against the MOWSE peptide mass fingerprint database (5). The remaining digested peptides (90% of total digest) were then reacted with N-succinimidyl-2(3-pyridyl) acetate (SPA) in order to enhance b-ion abundance and facilitate sequence analysis by tandem mass spectrometry (6). Dried peptide fractions were treated with 7 µl 1% w/v N-succinimidyl-2(3-pyridyl) acetate in 0.5M HEPES (pH 7.8 with NaOH) containing 15% v/v acetonitrile for 20 min on ice. The reaction was terminated by 1 µl heptafluorobutyric acid (HFBA) and the solution immediately injected onto a capillary reverse-phase column (300 µm x 15cm) packed with POROS R2/H material (Perseptive Biosystems, MA) equilibrated with 2% v/v acetonitrile/0.05% v/v TFA running at 3 µl/min. The absorbed peptides were washed isocratically with 10% v/v acetonitrile/0.05% v/v TFA for 30 minutes at 3 µl/min to elute the excess reagent and HEPES buffer. The derivatised peptides were then eluted with a single step gradient to 75% v/v acetonitrile/0.1% v/v formic acid and collected in a single 4 µl fraction. The derivatised peptides were then sequenced by low-energy collison-activated dissociation (CAD) using a Finnigan MAT TSQ7000 fitted with a nanoelectrospray source (7,8). CAD was performed using 2.5 mTorr argon with collisonal offset voltages between -18V and -28V. The product-ion spectra were collected with Q3 scanned at 500 amu/sec.

RESULTS:

Sequence data obtained from 7.5% gel (single band)

from GR78_human

Specific identifying peptides:

NQLTSNPENTVFDAK	82-96
SDIDEIVLVGGSTR	353-366
TWNDPSVQQDIK	107-113

Identified human protein GR 78:

Kd glucose related protein precursor (GRP78)

Immunoglobulin heavy chain binding protein (BIP)

Part 2: CLONING, SEQUENCING AND EXPRESSION OF p78

1) mRNA isolation and PCR amplification of identified genes.

Human chondrocytes were isolated and cultured for three weeks as described. Poly(A) mRNA (1-2μg) was extracted with a Micro-Fastrack kit (Invitrogen) from a total of 1-2 X 10^6 cells. One microgram of the resulting mRNA was reverse transcribed into cDNA in a 20μl volume at 45°C for 1 hour using 1μl of Moloney murine leukemia virus reverse transcriptase (200u/μl); 5 X first strand buffer (Tris-HCl pH 8.3, 250mM; KCl 375 mM; MgCl 15mM); 0.1M

DTT; oligo dT(12-18) 20ng/µl (Life Technologies); and dNTP mix 100mM (Amersham Pharmacia Biotec, Uppsala Sweden).

PCR was performed in a 50µl reaction volume under standard conditions (see below) using a Perkin Elmer Applied Biosystems thermal cycler PE2400. Primer sequences were derived from the GenBank database sequence corresponding to the Human gene for the immunoglobulin heavy chain binding protein, Bip (grp78), accession number X87949. Specific primers were synthesized to amplify the putative autoantigen gene from the chondrocyte cDNA. The resulting PCR product consisted of most of the grp78 coding region, bar the untranslated regions, signal sequences and the stop codon (nucleotide positions 279-2169 of the grp78 database sequence).

Primer sets for PCR were designed with integrated restriction sites to allow rapid subcloning of cDNA into the bacterial expression vector. The Forward primers encoded an NdeI site and the Reverse primers contained an XhoI restriction site: The Sequence Listing for the forward primer is given hereafter as SE4 and that for the reverse primer is given as SE5.

Bip Forward primer 5' TATACATATGGAGGAGGACAAGAAGGAGGACG 3' (32mer)
Bip Reverse primer 5' CCACCTCGAGTTCTGCTGTATCCTCTTCACCA 3' (32mer)

After initial denaturation at 96°C for 2min the, PCR was performed for 28 cycles using a cycling profile of 94°C for 30s, 60°C for 30s and 72°C for 2min,

with a final extension at 72°C for 7min. The PCR reaction generated a single specific Bip fragment of 1890bp.

2) Cloning of PCR generated fragments.

The restriction sites engineered into the forward and reverse primers used for the PCR reactions required flanking DNA for them to be recognised by their specific endonucleases (NdeI and XhoI). To provide this flanking DNA, the PCR generated fragment was cloned into a PCR cloning vector pCR2.1-TOPO (Invitrogen). The ligated plasmids were transformed into competent E.coli XL1-Blue (Stratagene) and plasmid DNA extracted using miniprep purification columns (Qiagen). The purified plasmid DNA for the clone was designated Bip-Topo. These DNA samples were stored at -20°C. The purified plasmid DNA for Bip-Topo was digested with NdeI and XhoI. The restricted fragments were separated by agarose gel electrophoresis and purified using the Qiagen DNA gel extraction kit.

3) Subcloning of restricted gene fragments into bacterial expression vector.

The purified fragment for the clone was ligated into the NdeI /XhoI pre-digested bacterial expression vector pET30a (Novagen). Ligation was performed at 12°C overnight in the presence of T4 ligase (20 units) and 1/10 vol of 10X ligase buffer (provided with the T4 ligase enzyme from Promega). The ligated plasmids were transformed into competent E.coli XL1-Blue (Stratagene) and screened by colony-PCR using Bip specific primers. Positive transformants carrying the required recombinant plasmids were purified and transformed into competent E.coli expression strain BL21-(DE3) (Invitrogen).

It will of course be understood that cloning may be carried out in prokaryotic or eukaryotic hosts including bacteria, insect cells, and mammalian cells. Preferred hosts are those which ensure glycosylation of the expressed product.

4) Sequencing of the 1890bp pET30::Bip subclone .

Sequencing of the 5' and 3' terminal ends of the pET30a::Bip clone confirmed that the recombinant DNA molecule was in-frame with the ATG start codon on the pET30 vector and that readthrough from this site continued through the Bip gene and ended with the 6X His residues and the stop codon located on the 3' arm of the expression vector.

Extensive DNA sequencing was performed using synthetic oligonucleotide primers spanning the entire length of the Bip subclone. Sequence analysis of the newly subcloned Bip gene fragment was performed by comparative alignment against the existing grp78 sequence from the database (accession number X87949). A number of differences between the two sequences were detected, both at the DNA and protein level (see Appendix 2). These areas of disagreement may either be a result of errors in the original DNA sequencing (of grp78) or they may indicate the presence of an additional related, but slightly different Bip gene in the genome.

All DNA sequencing was performed on an Applied Biosystems ABI 377 automated DNA sequencer using the dRhodamine dye terminator kit (Perkin Elmer- Applied Biosystems).

Expression of bacterial and purification recombinant proteins.

E.coli expression strain BL21-(DE3) containing the recombinant pET30a-Bip plasmid was grown at 37°C in LB medium containing kanamycin (50μg/ml). When the cells had reached an OD600 of 0.6units, isopropyl β-D-thiogalactopyranoside (IPTG) (1mM) was added to the medium to induce expression of the recombinant protein, driven by the IPTG-inducible promoter of the expression vector. To allow for maximal expression of the recombinant protein the culture was incubated for a further 4 hours at 37°C. The cells were pelleted by centrifugation and stored at -70°C.

For purification of the recombinant bacterial proteins the bacterial pellets were lysed in binding buffer (20mM NaPO4, 500mM NaCl. 5mM Imidazole, 1mM PMSF, 1mg/ml Lysozyme, 5units/ml DNAse, 0.1% Triton X-100, pH7.4). The lysate was cleared by centrifugation to remove insoluble matter and cell debris. The cleared lysate was passed over a binding buffer-equilibrated chelating Hi-trap affinity column, with a bed volume of 5ml (Pharmacia). The non-specifically bound protein was washed from the column under stringent conditions using a series of three wash buffers. The primary wash was performed using 100ml of Binding buffer. This was followed by a high stringency low pH wash (20mM NaPO4, 500mM NaCl, 0.1% Triton X-100, pH5.5) and an additional high stringency wash using 100ml of 20mM NaPO4, 500mM NaCl, 0.1% Triton X-100, 50mM Imidazole, pH7.4.

The histidine-tagged recombinant proteins were eluted from the column by stripping with 50mM EDTA. Eluted proteins were dialysed overnight against 1x PBS to remove EDTA and Ni contaminants. The purified protein was

concentrated and washed in sterile PBS using a 50000 Mw cutoff concentrator column (Millipore). The total amount of protein was determined by spectrophotometry using BSA as a standard with the bicinchoninic acid assay.

Immunological studies in experimental arthritis

Antibody response to p78 in experimental arthritis

Collagen arthritis (CIA) and pristane arthritis (PIA) were induced in DBA/1 mice according to our previously described protocol. Mice were bled before induction of arthrtis (15 animals), at the onset of CIA (16 animals) and the onset of PIA (14 animals). The antibody in mouse sera against p78 was determined using an enzyme-linked immunosorbent assay (ELISA) with recombinant p78. Nunc 96-well ELISA plates (Fisher Biotech, Orangeburg, NY) were coated overnight at 4^0C with p78 at 500 ng (in 100 ml of 5% non-fat milk/PBS) per well. After washing 3 times with phosphate buffered saline (PBS) containing 0.05% Tween-20, the plate was blocked with 5% non-fat milk/PBS overnight at 4^0C. The mouse sera were added into the wells at 1:200 dilution in milk/PBS and incubated overnight at 4^0C. The plate was washed, 100 ml goat anti-mouse Ig conjugated with alkaline phosphatase (anti-Ig-AK: 1:500 dilution in milk/PBS/Tween-20, Fisher Biotec) was added for 60 mins at 37^0C. After three washes with PBS/Tween-20, 100 ml paranitrophenyl phospphate solution (PNPP tablets; Sigma Chemicals; St Louis, MO) in diathylanolamine buffer was added to each well. The reaction was allowed to proceed for 30 mins in the dark and the plate read at 405nm in aspectrophotometer (Molecular Devices, Menlo Park, CA). The data were analyzed using the SOFTmax analytical software package. The specific

binding were the OD readings from p78-coated wells subtractinf the OD from non-coated as well as non-sera blanks. The antibody levels were expressed as OD_{405} units.

RESULTS

Identification of autoantigen

When RA and control sera were blotted against chondrocyte extracts, 30% RA sera reacted with a 78 Kd protein compared to 10% of control sera (Figure 1). Sequencing of three tryptic peptides by low energy CAD identified one component of the 78 kD band as the 78 kD glucose-regulated protein, also known as immunoglobulin heavy chain binding protein (BiP). DNA sequence analysis of p78 from articular chondrocyte cDNA showed a number of deviations from the published sequence (accession number X87949). A total of six single nucleotide substitutions and a codon insertion result in three amino acid substitutions and an arginine insertion at position 834-836 of p78 (accession number AF188611).

Immunological tests in rheumatoid arthritis

T cell proliferative responses were determined for mononuclear cell preparations from paired peripheral blood and synovial fluid samples obtained from 23 patients with rheumatoid arthritis and from 12 disease controls. Twelve of 23 (52 per cent) patients with RA and only 2 of 12 (17 per cent) of disease controls showed increased synovial proliferation (Figure 2). The prolifeative response to p78 of RA synovial T cells was significantly higher than that of the paired peripheral blood (stimulation index, mean ± SEM: SF 3.5 ± 0.7; PB 1.6 ± 0.2; $p < 0.01$ Wilcoxon paired test)). A significant difference was also seen between synovial fluid responses to p78 between RA

patients and disease controls (SI: RA 3.5 ± 0.7; OIJD 1.4 ± 0.2; p=0.03 Mann Whitney U test). There was no association with HLA-DR as 50% of responders and non-responders were HLA-DR4 positive (data not shown).

Rheumatoid synovial fluid T cell proliferation to p78 was inhibited by 66-84% by anti-HLA-DR monoclonal antibody L243 (ATCC, Rockville, MD) (data not shown).

No IFNg could be measured in the supernatants from the paired synovial fluid and peripheral blood mononuclear cells (data not shown) despite using an ELISA sensitive to 0.01 ng/ml. No IFNg could be detected by intracellular fluorescence in the peripheral blood T cells of RA patients (n = 7) or healthy controls (n = 2) after stimulation by p78 (data not shown). These findings imply that the responding T cells were unlikely to belong to the classical IFNg producing TH1 subset{1461}.

Immunological studies in experimental arthritis

Induction of experimental arthritis with p78

Immunisation of DBA/1, C57BL mice and Lewis rats with p78 in Freund's complete adjuvant (CFA) did not lead to the development of arthritis (data not shown). There was a similar lack of arthritogenicity of p78 when injected with CFA into HLA-DR1$^{+/+}$ (0/10 mice) or into HLA-DR4$^{+/-}$ (0/5) mice.

Immune response to p78 in experimental arthritis

Despite the failure to induce arthritis by immunising animals with p78, we investigated whether DBA/1 mice during the course of collagen (CIA) or pristane (PIA) induced arthritis developed antibodies to p78 (Figure 4). Mice developed serum anti-p78 antibodies at the onset of collagen arthritis ($O.D_{405}$.

0.189 ± 0.042, m ± sem) and pristane induced arthritis (0.504 ± 0.074) when compared to pre-bleed sera (0.070 ± 0.019; p < versus CIA and p< versus PIA, respectively). Furthermore the concentration of these antibodies was significantly higher in the PIA mice as compared to CIA mice (p). There were 14 mice in each group.

Prevention of collagen-induced arthrtis bv intravenous administration of p78

The presence of antibodies to p78 in the sera of mice with CIA or PIA suggested that manipulating the immune response to p78 might prevent the subsequent development of CIA by a bystander phenomenon. HLA-DR1$^{+/-}$ transgenic mice were injected intravenously with 1mg of p78 prior to immunisation with type II collagen in CFA one week later (Table 2). Whereas 83% of animals had 46% of their limbs involved with arthritis at 8 weeks when pretreated with saline, only 10% of animals had 3% of their limbs involved with arthritis in the group previously given intravenous p78. These differences are highly significant ($p \leq 0.008$ and $p \leq 0.0001$). Table 2 also shows that there was a significant reduction in anticollagen antibodies in the p78 pre-treated animals to one third the level in the controls. The reduction was equal in the the IgG1 and IgG2 isotypes (Table 3). The histology of the joints of these animals (Figure 6) confirmed the clinical findings in that there was no synovitis in the joints of p78 pre-treated mice.

We have shown in this study that 30% of patients with RA have antibodies, detected by Western blotting, directed against the human chaperonin BiP/GRP78, and named by us p78. Furthermore, T cells from the rheumatoid synovial fluid proliferated preferentially to p78. There was minimal response by the T cells from the peripheral blood of the same patients. The T cells from

patients with other inflammatory arthritides, whether from the synovial fluid or the peripheral blood, did not significantly proliferate to p78. Finally, the proliferative response was inhibited by anti-HLA-DR monoclonal antibody suggesting that CD4+ positive T cells are responding to antigenic peptides presented in the context of HLA-DR.. This polyclonal T cell response was not HLA-DR4 restricted. Thus p78 stimulation of T cell proliferation possesses two characteristics to be expected of a rheumatoid autoantigen, namely, it is joint and disease specific.

On the basis of these observations, we then undertook intravenous immunisation of mice with p78 in order to test the hypothesis that deviating the immune response to p78 would prevent the development of CIA by a bystander mechanism.. This indeed proved to be the case with almost total prophylaxis against the induction of CIA in HLA-DR1$^{+/+}$ transgenic mice. In the past, various types of experimental arthritis have been prevented or treated by administration of bacterial, and especially mycobacterial, heat shock proteins such as HSP60 or T cells responding to whole HSP60 or to specific peptides{2103, 2280,2282,2283,2284,2281}. However, it is of some importance to note that self-HSP60 peptides show no such protective effects{2281}. Thus the ability of p78 to prevent CIA is of fundamental importance. The observations described in this work are the first, to our knowledge, which implicate an endogenous chaperone in the pathogenesis of RA and the immunotherapy of experimental arthritis. The potential for the immunotherapy of RA is clearly apparent.

2. Use of tests for detection of antibodies to p78 in biological fluids or culture supernatants

Several techniques can be used, such as agglutination,Western blotting, and ELISA.

ELISA protocol for the detection of antibodies to p78 in sera

ELISA plates are coated half with p78 in bicarbonate buffer and half with bicarbonate buffer alone for 4 hours at room temperature. After 2 washes in PBS the plate is blocked for 2 hours at room temperature with 10% goat serum in PBS with 0.05% Tween 20 to stop non-specific binding of protein. After 2 further washes diluted sera (in PBS/1% goat serum/0.05%Tween) is added in duplicate to both the p78 coated and non-coated sides of the plate. After 4 washes biotin conjugated anti human immunoglobulin (1/10000 diluted in PBS/1% goat serum/0.05% Tween)) is added to the plate. The plate is washed 6 times. Bound biotinylated antibody is detected with streptavidin conjugated horse-radish peroxidase (1/800 inPBS/1% goat serum/0.05% Tween) and suitable substrate. Sera containing antibodies to p78 are determined spectrophotometrically. This test forms the basis of a diagnostic and/or prognostic test for rheumatoid arthritis.

3. Therapeutic application

Many routes of administration of the recombinant protein or vector are possible, including intravenous, intramuscular, nasal, oral, cutaneous, and topical. There are several approaches to using p78 or derivatives for therapeutic purposes, including the following:

(a) Induction of mucosal tolerance.

Delivery of p78 autoantigen or peptides derived therefrom by mucosal routes, e.g. through the intestine or nasal mucosa, alters the immune response by downregulating disease activity leaving the patient's immune system otherwise intact. Alternatively p78 or p78 peptides can be delivered as DNA plasmids encoding them with an appropriate mammalian expression vector.

(b) Vaccination with TCR peptides

Peptides of the CDR3 region of the T cell receptor Valpha and Vbeta chains can be synthesised and used as vaccines for delivery by intradermal or intramuscular injection. Plasmids encoding these peptides can be used in the same way.

(c) MHC blockade with native or altered peptides

The p78 peptides may be given parenterally or orally in appropriate cases either unmodified or modified by amino acid substitution and/or attachment of chemical groupings so as to block MHC and especially HLA-DR4 thereby leading to suppression of T cell activation and disease. P78 peptides either native or altered may be combined with soluble HLA-DR4 molecules and applied parenterally or orally.

(d) Induction of tolerance by plasmid DNA immunisation

Plasmids consisting of DNA coding for whole p78 protein or its peptides linked to a mammalian expression vector may be given by injection. DNA coding for human IL-10, IL-4, IL-11, or TGF-beta, incorporated singly or in any combination, may be used to deviate the immune response to p78 towards a TH2 mode so as to suppress disease.

In the therapeutic regimes indicated above the protein or derived peptide may be administered in appropriate compositions delivering amounts ranging from about 0.1 micrograms to about 1 gram or the equivalent in the case of plasmid or vaccine preparations.

Appendix 1

Methodology for gel electrophoresis

Acrylamide gel: 10%

6.075ml acrylamide (40%) (BDH, Poole, UK)

3.35ml methylenbisacrylamide (2%)(Pharmacia Biotech, Uppsala, Sweden)

6.25ml acrylamide gel buffer (see below)

9ml distilled water (produced within the laboratory)

250µl Ammonium persulphate (AMPERS)(0.025mg in 250ul of distilled water)(Sigma-Aldrich, Poole, UK)

25µl NNN'N'-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Poole, UK)

Acrylamide gel buffer: pH 8.8

1.5M Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

pH titration with concentrated hydrochloric acid

0.4% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Stacking gel:

1.2ml acrylamide (40%)(BDH-Merck, Poole, UK)

0.65ml bisacrylamide (2%)(Pharmacia Biotech, Uppsala, Sweden)

3.15ml stacking gel buffer (see below)

7.5ml distilled water (produced within the laboratory)

125µl ammonium persulphate (AMPERS) (0.025mg/250µl)(Sigma-Aldrich, Poole, UK)

12.5µl NNN'N'-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Poole, UK)

Stacking gel buffer: pH 6.8:

0.5M Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

pH titration with concentrated hydrochloric acid

0.4% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Loading buffer:

2ml glycerol

2ml 10% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

0,.25mg bromophenol blue

2.5ml stacking gel buffer 4-times concentrated (0.5M Tris; 0.4% SDS; pH 6.8)

0.5ml 2-mercaptoethanol (Sigma-Aldrich, Poole, UK)

Electrophoresis/Running buffer:

3g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

14.4g/l glycine (BDH-Merck, Poole, UK)

1g/l Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Transfer buffer:

3g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

14.4g/l glycine (BDH-Merck, Poole, UK)

1g/l Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

14.4g/l glycine (BDH-Merck, Poole, UK)

10% methanol (BDH-Merck, Poole, UK)

Tris Tween buffered saline (TTBS):

2.4g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

29g/l sodium chloride (BDH-Merck, Poole, UK)

500µl tween 20 (polyoxyethylene-sorbitan mono-laurate)(Sigma-Aldrich, Poole, UK)

3% Bovine serum albumin solution (BSA):

3g Albumin bovine fraction 5 (BDH-Merck, Poole, UK)

100ml TTBS (see above)

Appendix 2

Amino acis sequence of the expressed BiP recombinant human protein. Total 1917 nucleotides. Starting at Methionine (start codon) ending in 6X His tag.

Molecular Weight 70937.50 Daltons
639 Amino Acids
86 Strongly Basic(+) Amino Acids (K,R)
108 Strongly Acidic(-) Amino Acids (D,E)
204 Hydrophobic Amino Acids (A,I,L,F,W,V)
142 Polar Amino Acids (N,C,Q,S,T,Y)

5.173 Isolectric Point
-20.041 Charge at PH 7.0

MEEDKKEDVGTVVGIDLGTTYSCVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRL
IGRTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVLTKMKETAEAYLGKKVTHAVVTVPAYFND
AQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGDTHLGGEDF
DQRVMEHFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKRALSSQHQARIEIESFYEGEDFSETLTRAKFEELNMDLFRST
MKPVQKVLEDSDLKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQAGVLSGDQDTGDLVLLD
VCPLTLGIETVGGVMTKLIPRNTVVPTKKSQIFSTASDNQPTVTIKVYEGERPLTKDNHLLGTFDLTGIPPAPRGVPQIE
VTFEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVNDAEKFAEEDKKLKERIDTRNELESYAYSLKNQIGD
KEKLGGKLSSEDKETMEKAVEEKIEWLESHQDADIEDFKAKKKELEEIVQPIISKLYGSAGPPPTGEEDTAELHHHHHH

Appendix 3

RT-PCR Cloned and Sequenced grp78 (BiP) fragment used for expression of recombinant human protein. Total 1917 nucleotides. Starting at ATG start codon ending in 6X His tag.

ATGGAGGAGGACAAGAAGGAGGACGTGGGCACGGTGGTCGGCATCGACCTGGGGACCACCTACTCCTGCGTCGGCGTGTT
CAAGAACGGCCGCGTGGAGATCATCGCCAACGATCAGGGCAACCGCATCACGCCGTCCTATGTCGCCTTCACTCCTGAAG
GGGAACGTCTGATTGGCGATGCCGCCAAGAACCAGCTCACCTCCAACCCCGAGAACACGGTCTTTGACGCCAAGCGGCTC
ATCGGCCGCACGTGGAATGACCCGTCTGTGCAGCAGGACATCAAGTTCTTGCCGTTCAAGGTGGTTGAAAAGAAAACTAA
ACCATACATTCAAGTTGATATTGGAGGTGGGCAAACAAAGACATTTGCTCCTGAAGAAATTTCTGCCATGGTTCTCACTA
AAATGAAAGAAACCGCTGAGGCTTATTTGGGAAAGAAGGTTACCCATGCAGTTGTTACTGTACCAGCCTATTTTAATGAT
GCCCAACGCCAAGCAACCAAAGACGCTGGAACTATTGCTGGCCTAAATGTTATGAGGATCATCAACGAGCCTACGGCAGC
TGCTATTGCTTATGGCCTGGATAAGAGGGAGGGGGAGAAGAACATCCTGGTGTTTGACCTGGGTGGCGGAACCTTCGATG
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Appendix 4

RT-PCR Cloned and Sequenced grp78 (BiP) fragment used for expression of recombinant human protein. Total 1917 nucleotides. Starting at ATG start codon ending in 6X His tag.□□ATGGAGGAGGACAAGAAGGAGGACGTGGGCACGGTGGTCGGCATCGACCTGGGGACC
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Appendix 4 (contd)

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aa

Table 1. Prevention of CIA by intravenous injection of recombinant p78

Tolerogen[a]	% Arhritic Mice at 8 weeks[b]	% Arthritic Limbs at 8 weeks[b]	Antibodies (IgG) to CII[c]
p78 (1mg)	1/10 (10%)*	1/40 (3%)**	22 ± 9***
PBS	5/6 (83%)	11/24 (46%).	68 ± 10

[a] HLA-DR1$^{+/+}$ transgenic mice were injected intravenously with either PBS (negative control) or recombinant p78. Either 1mg of protein dissolved in 0.1ml of PBS or 0.1ml of PBS was administered intravenously and mice were immunised with type II collagen in CFA seven days after the intravenous dose.

[b] The incidence of arthritis is reported at 8 weeks after immunisation.

[c] Antibodies represent mean units per group using sera collected 8 weeksafter immunisation.ELISAs were performed and results are reported as units of aactivity derived by cmparison of test sera with the standard serum which was arbitrarily defined as having 50 units of acitivity. Sera were analysed individually and results shown as the mean ± SD for each group of animals.

* $p \leq 0.008$ (Fischer's Exact test) ** ≤ 0.0001 (Fischer's Exact test) *** <0.05 (Students t test)

Table 2. IgG1 and IgG2 antibody isotypes to type II collagen in mice treated intravenously with either recombinant p78 or PBS.

Tolerogen [a]	IgG1 antibodies to type II collagen [b]	IgG2 antibodies to type II collagen [b]
p78 (1000 μg)	0.71 ± 0.019 *	0.110 ± 0.022 *
PBS	0.135 ± 0.066	0.250 ± 0.044

a HLA-DR1 transgenic mice were injected intravenously with either PBS (negative control) or recombinant p78 followed by immunisation with collagen type II in CFA one week later .

b ELISAs were performed as described in caption to Table 2.

*$p<0.05$ (Students t test)

Table 3: Anti-p78 antibody in CIA or PIA mouse sera (1:200 dil)

Subtract blanks and non-coated values			
Mouse ID	Prebleed	CIA onset	PIA(mouse ID#1-14)
961	0.000	0.243	0.263
982	0.181	0.328	0.981
963	0.155	0.567	0.780
965	0.076	0.198	0.934
966	0.162	0.257	0.388
967	0.068	0.000	0.291
968	0.039	0.189	0.469
970	0.000	0.000	0.551
197	0.173	0.000	0.537
198	0.023	0.000	0.711
200	0.000	0.099	0.430
248	0.000	0.183	0.535
702	0.090	0.011	0.038
703	0.012	0.206	0.147
705		0.341	
706		0.407	
Mean	0.070	0.189	0.504
Count	14	16	14
SEM	0.019	0.042	0.074

Legends to figures

Figure 1
Western blotting showing 6 rheumatoid sera (lanes 1-6), 5 normal sera (lanes 7-11) and 4 disease controls (lanes 12-15) reacting with chondrosarcoma lysate. Molecular weight markers are shown.

Figure 2
Lymphocyte proliferation in mononuclear cells cultured for 6 days expressed as a stimulation index: proliferation in the presence of p78/proliferation in the presence of culture medium alone. A stimulation of ≥ 2.5 was considered significant. RAPB, rheumatoid arthritis peripheral blood; RASF, rheumatoid arthritis synovial fluid; OIJDPB, other inflammatory joint diseases peripheral blood; OIJDSF, other inflammatory joint diseases synovial fluid.

Figure 3
Antibodies to recombinant human p78 in the sera of mice measured by ELISA and expressed as OD_{405}. Shown are the values for the animals bled before the induction of experimental arthritis (pre-bleed), and at the onset of collagen- induced arthritis (CIA) and of pristane-induced arthritis (PIA).

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CLAIMS

1. The use of immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom, as a reagent for indicating the presence of rheumatoid arthritis.

2. The use of protein BiP(78KD) according to claim 1, in which the protein has the amino-acid sequence of Sequence Listing SE1 or SE2.

3. The protein BiP(78KD) having the amino-acid sequence of Sequence Listing SE1 or SE2..

4. Recombinant BiP(78KD).

5. A prognostic or diagnost test for RA in which the test reagent for testing body fluid is immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom.

6. A test according to claim 4, being an ELISA assay.

7. A test according to claim 4, being a Western Blot.

8. Immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom, for use in therapy.

9. Immunoglobulin heavy chain binding protein BiP (78KD), or a peptide derived therefrom, for use in rheumatoid arthritis therapy.

10. A DNA sequence coding for Immunoglobulin heavy chain binding protein BiP (78KD), or for a peptide derived therefrom, for use in therapy.

11. A DNA sequence coding for Immunoglobulin heavy chain binding protein BiP (78KD), or for a peptide derived therefrom, for use in rheumatoid arthritis therapy.

12. A DNA sequence having or containing the nucleotide sequence of Sequence Listing SE3.

13. A recombinant vector containing a DNA sequence according to claim 12.

14. A method of testing for RA, using a protein BiP(78KD) or a cDNA sequence coding therefor to determine the presence of antibodies to said protein.

15. A method of therapeutic treatment of RA comprising administering the protein BiP(78KD) or a DNA coding therefor.

16. A method according to claim 15, in which administration is by the intravenous, nasal, oral or cutaneous route.

17. A recombinant organism capable of expressing protein BiP (78KD).

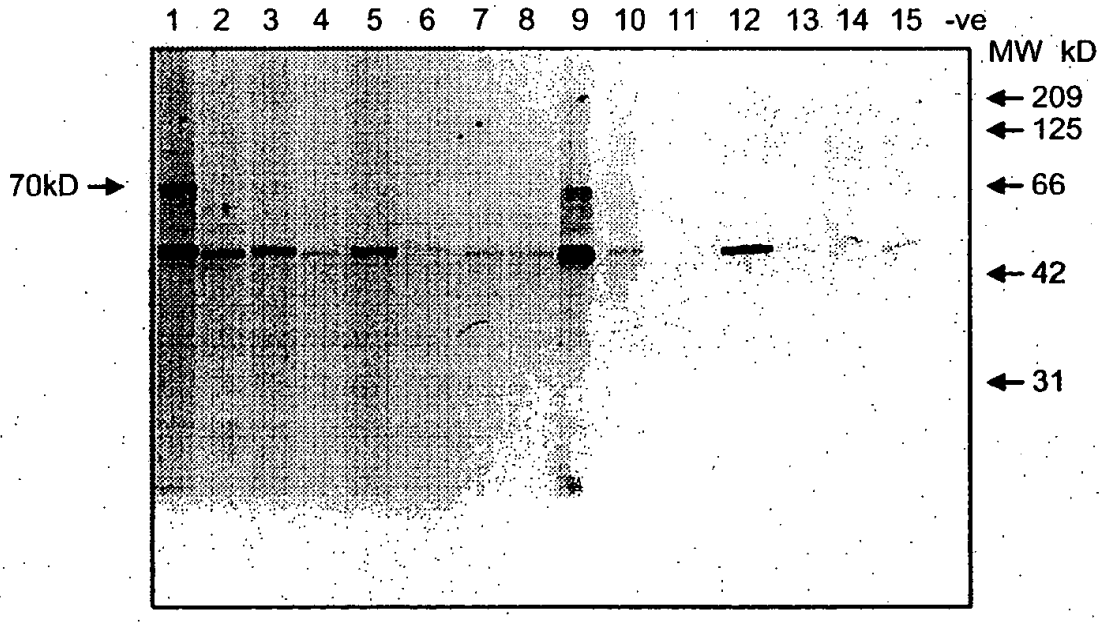

FIG. 1

FIG. 2

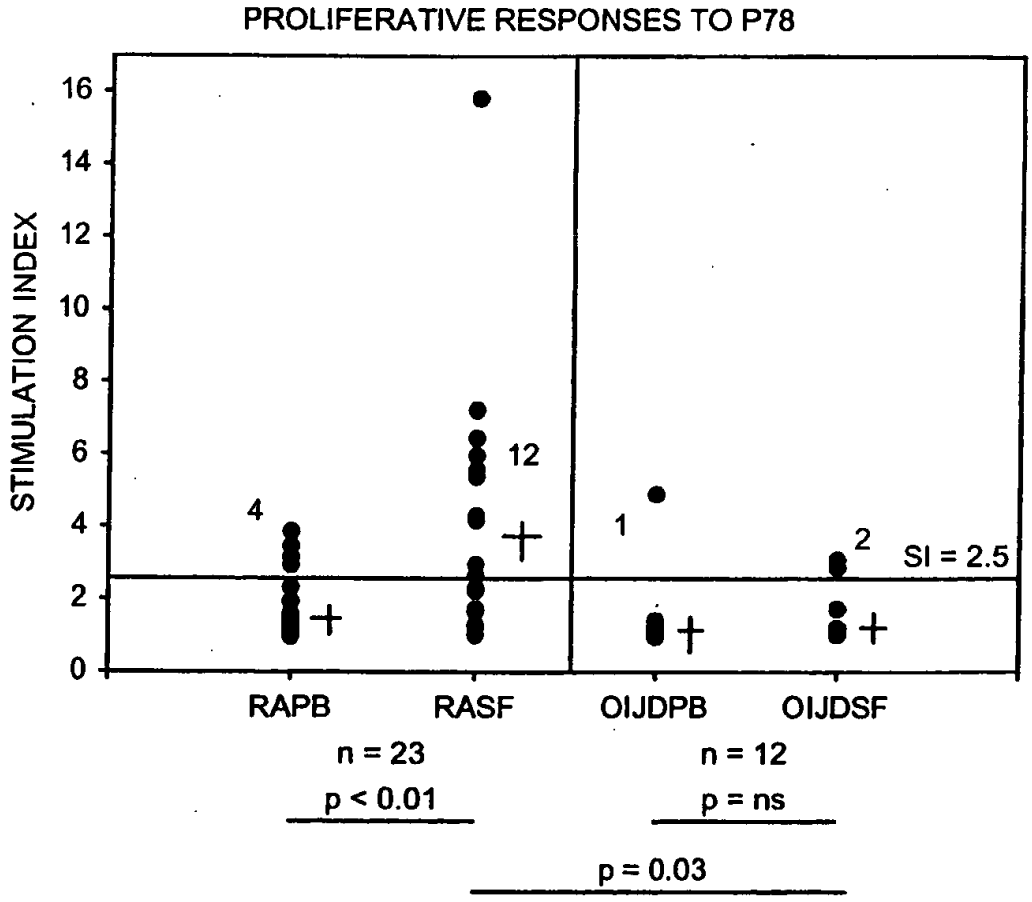
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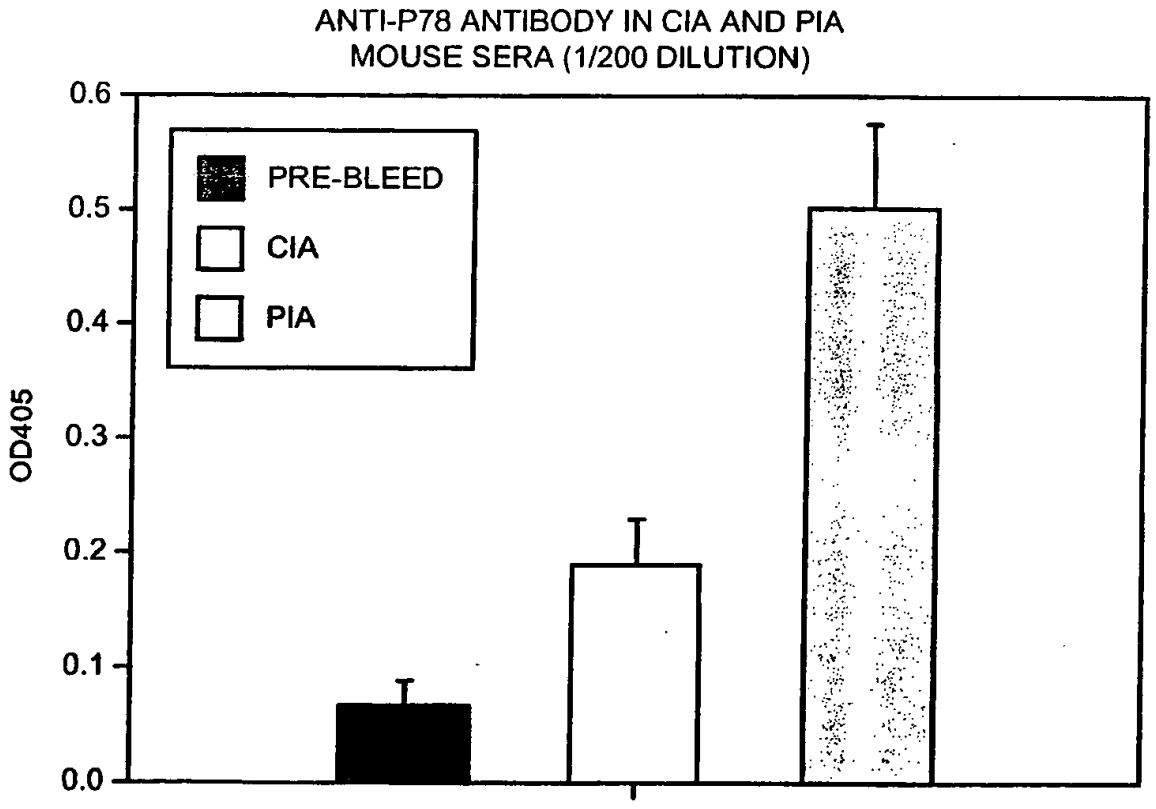
FIG. 3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Lys Leu Ile Pro Arg Asn Thr Val Val Pro Thr Lys Lys Ser Gln Ile
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Phe Ser Thr Ala Ser Asp Asn Gln Pro Thr Val Thr Ile Lys Val Tyr
435 440 445

Glu Gly Glu Arg Pro Leu Thr Lys Asp Asn His Leu Leu Gly Thr Phe
450 455 460

Asp Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu
465 470 475 480

Val Thr Phe Glu Ile Asp Val Asn Gly Ile Leu Arg Val Thr Ala Glu
485 490 495

Asp Lys Gly Thr Gly Asn Lys Asn Lys Ile Thr Ile Thr Asn Asp Gln
500 505 510

Asn Arg Leu Thr Pro Glu Glu Ile Glu Arg Met Val Asn Asp Ala Glu
515 520 525

Lys Phe Ala Glu Glu Asp Lys Lys Leu Lys Glu Arg Ile Asp Thr Arg
530 535 540

Asn Glu Leu Glu Ser Tyr Ala Tyr Ser Leu Lys Asn Gln Ile Gly Asp
545 550 555 560

Lys Glu Lys Leu Gly Gly Lys Leu Ser Ser Glu Asp Lys Glu Thr Met
565 570 575

Glu Lys Ala Val Glu Glu Lys Ile Glu Trp Leu Glu Ser His Gln Asp
580 585 590

Ala Asp Ile Glu Asp Phe Lys Ala Lys Lys Lys Glu Leu Glu Glu Ile
595 600 605

Val Gln Pro Ile Ile Ser Lys Leu Tyr Gly Ser Ala Gly Pro Pro Pro

610 615 620

Thr Gly Glu Glu Asp Thr Ala Glu Leu His His His His His His
625 630 635

<210> 2
<211> 633
<212> PRT
<213> Homo sapiens

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Leu Gly Thr Thr Tyr Ser Cys Val Gly Val Phe Lys Asn Gly Arg Val
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Glu Ile Ile Ala Asn Asp Gln Gly Asn Arg Ile Thr Pro Ser Tyr Val
35 40 45

Ala Phe Thr Pro Glu Gly Glu Arg Leu Ile Gly Asp Ala Ala Lys Asn
50 55 60

Gln Leu Thr Ser Asn Pro Glu Asn Thr Val Phe Asp Ala Lys Arg Leu
65 70 75 80

Ile Gly Arg Thr Trp Asn Asp Pro Ser Val Gln Gln Asp Ile Lys Phe
85 90 95

Leu Pro Phe Lys Val Val Glu Lys Lys Thr Lys Pro Tyr Ile Gln Val
100 105 110

Asp Ile Gly Gly Gly Gln Thr Lys Thr Phe Ala Pro Glu Glu Ile Ser
115 120 125

Ala Met Val Leu Thr Lys Met Lys Glu Thr Ala Glu Ala Tyr Leu Gly
130 135 140

Lys Lys Val Thr His Ala Val Val Thr Val Pro Ala Tyr Phe Asn Asp
145 150 155 160

Ala Gln Arg Gln Ala Thr Lys Asp Ala Gly Thr Ile Ala Gly Leu Asn
165 170 175

Val Met Arg Ile Ile Asn Glu Pro Thr Ala Ala Ala Ile Ala Tyr Gly
180 185 190

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Leu Asp Lys Arg Glu Gly Glu Lys Asn Ile Leu Val Phe Asp Leu Gly
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Gly Gly Thr Phe Asp Val Ser Leu Leu Thr Ile Asp Asn Gly Val Phe
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Glu Val Val Ala Thr Asn Gly Asp Thr His Leu Gly Gly Glu Asp Phe
225                 230                 235                 240

Asp Gln Arg Val Met Glu His Phe Ile Lys Leu Tyr Lys Lys Lys Thr
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Gly Lys Asp Val Arg Lys Asp Asn Arg Ala Val Gln Lys Leu Arg Arg
            260                 265                 270

Glu Val Glu Lys Ala Lys Arg Ala Leu Ser Ser Gln His Gln Ala Arg
        275                 280                 285

Ile Glu Ile Glu Ser Phe Tyr Glu Gly Glu Asp Phe Ser Glu Thr Leu
    290                 295                 300

Thr Arg Ala Lys Phe Glu Glu Leu Asn Met Asp Leu Phe Arg Ser Thr
305                 310                 315                 320

Met Lys Pro Val Gln Lys Val Leu Glu Asp Ser Asp Leu Lys Lys Ser
                325                 330                 335

Asp Ile Asp Glu Ile Val Leu Val Gly Gly Ser Thr Arg Ile Pro Lys
            340                 345                 350

Ile Gln Gln Leu Val Lys Glu Phe Phe Asn Gly Lys Glu Pro Ser Arg
        355                 360                 365

Gly Ile Asn Pro Asp Glu Ala Val Ala Tyr Gly Ala Ala Val Gln Ala
    370                 375                 380

Gly Val Leu Ser Gly Asp Gln Asp Thr Gly Asp Leu Val Leu Leu Asp
385                 390                 395                 400

Val Cys Pro Leu Thr Leu Gly Ile Glu Thr Val Gly Gly Val Met Thr
                405                 410                 415

Lys Leu Ile Pro Arg Asn Thr Val Val Pro Thr Lys Lys Ser Gln Ile
            420                 425                 430

Phe Ser Thr Ala Ser Asp Asn Gln Pro Thr Val Thr Ile Lys Val Tyr
        435                 440                 445
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Glu Gly Glu Arg Pro Leu Thr Lys Asp Asn His Leu Leu Gly Thr Phe
450 455 460

Asp Leu Thr Gly Ile Pro Pro Ala Pro Arg Gly Val Pro Gln Ile Glu
465 470 475 480

Val Thr Phe Glu Ile Asp Val Asn Gly Ile Leu Arg Val Thr Ala Glu
485 490 495

Asp Lys Gly Thr Gly Asn Lys Asn Lys Ile Thr Ile Thr Asn Asp Gln
500 505 510

Asn Arg Leu Thr Pro Glu Glu Ile Glu Arg Met Val Asn Asp Ala Glu
515 520 525

Lys Phe Ala Glu Glu Asp Lys Lys Leu Lys Glu Arg Ile Asp Thr Arg
530 535 540

Asn Glu Leu Glu Ser Tyr Ala Tyr Ser Leu Lys Asn Gln Ile Gly Asp
545 550 555 560

Lys Glu Lys Leu Gly Gly Lys Leu Ser Ser Glu Asp Lys Glu Thr Met
565 570 575

Glu Lys Ala Val Glu Glu Lys Ile Glu Trp Leu Glu Ser His Gln Asp
580 585 590

Ala Asp Ile Glu Asp Phe Lys Ala Lys Lys Lys Glu Leu Glu Glu Ile
595 600 605

Val Gln Pro Ile Ile Ser Lys Leu Tyr Gly Ser Ala Gly Pro Pro Pro
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Thr Gly Glu Glu Asp Thr Ala Glu Leu
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<212> DNA
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<212> DNA
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<220>
<223> Description of Artificial Sequence: primer

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<210> 5
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<212> DNA
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<220>
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